Magnesium deficiency heightens lipopolysaccharide-induced inflammation and enhances monocyte adhesion in human umbilical vein endothelial cells

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Abstract

Given a possible anti-inflammatory role of magnesium in endothelial cells, the aim of this study was to investigate the effects of magnesium on human umbilical vein endothelial cell (HUVEC) viability, gene expression, and the pro-inflammatory response caused by a bacterial endotoxin (LPS). HUVECs were cultured at three different concentrations of magnesium sulphate (0.1mM; control-1mM; 5mM) for 72 hours. Exposing the cells to LPS reduced cell viability in culture with low magnesium, but high magnesium protected the HUVECs from LPS-induced cell death. LPS-treated HUVECs cultured in low magnesium showed up-regulation of mRNA expression for pro-inflammatory factors and the expression of cytokine proteins, including IL-2, IL-3, IL-8, IL-15 and MCP-1. This was associated with greater adhesion of monocytes to the cells. In contrast, high magnesium decreased the expression of inflammatory factors and cytokines. The study found that LPS activation of the expression of many pro-inflammatory factors is exacerbated in the presence of low magnesium concentration whilst a high magnesium concentration partly inhibited the inflammatory response to LPS.

Introduction

Endothelial cells are present along the length of all blood vessels and control the movement of macroparticles and hormones and regulate blood flow [1], cellular adhesion, vascular inflammation, vessel tone and smooth muscle proliferation [2]. Endothelial dysfunction plays a role in many vascular diseases, such as thrombosis and atherosclerosis. In atherosclerosis, plaques develop in the walls of the arteries in response to the dysfunction of endothelial cells, which express cytokines and adhesion molecules that recruit T-cells and drive monocytes and macrophages to the sub-endothelial space [3].

Epidemiological studies suggest that poor intakes of magnesium are associated with greater risk of cardiovascular disease [4]. These studies are complemented by animal studies that have demonstrated magnesium supplementation to limit the development of atherosclerotic lesions in LDL receptor and apolipoprotein E knockout mice [5, 6] Magnesium status may impact on cardiovascular health through a number of mechanisms [7]. Previous work suggests that low extracellular magnesium concentrations have a negative impact on endothelial cell proliferation, increase monocyte adhesion, inhibit cell migration and markedly alter endothelial cell gene expression. Low magnesium levels may also contribute to inflammation. These effects may be due to the activation of many types of cytokines, which induce an overexpression of the inflammatory phenotype in endothelial cells [8]. The current study explored the hypothesis that the expression of adhesion molecules and cytokines would be enhanced in human umbilical vein endothelial cells (HUVECs) cultured under conditions of magnesium depletion, and that the enhanced inflammatory response would increase monocyte adhesion, which may represent an early stage in atherosclerosis.

Methods and Materials

Cell Culture

Primary HUVECs (C2519A; Lonza Basel, Switzerland) were cultured in endothelial cell growth medium (EGM-2, Lonza) with 2% foetal bovine serum (FBS). Cells were incubated in six well plates at seed density (7500/cm^2), at 37°C (5% CO_2), with medium changed every other day until the cells were grown to 80–90% confluence. At 80% confluence, cells were transferred to human endothelial Mg-free medium (Invitrogen, USA), supplemented with 10% FBS, 1% penicillin 100× 1%, 5% endothelial cell growth supplement (Sigma-Aldrich, UK), and magnesium sulphate ($MgSO_4$) concentrations of 0.1 mM (low Mg) and 5 mM (high Mg) following the method of Ferre *et al*., and Maier *et al*., [9, 10]. The samples were compared with cells cultured with 1 mM $MgSO_4$, which is the physiological circulating Mg concentration. After 3 days, the cells were treated for 4 h with 0.1 or 0.5 µg/mL lipopolysaccharide (LPS, *Salmonella enterica* serotype Enteritidis, Sigma-Aldrich) and the response was compared with untreated cells. Separate experiments were performed in order to obtain cell lysates and medium for the measurement of each component of the study (cell viability, adhesion molecule and cytokine mRNA expression, adhesion molecule and protein expression, cell adhesion).

For the cell viability assay, cell suspensions were mixed with 10 µl of 0.4% Trypan blue solution (Sigma Aldrich, UK) and pipetted onto a haemocytometer. The cells were examined under the microscope, and the number of viable and nonviable cells was counted.

Cell Adhesion Assay

The Vybrant™ Cell Adhesion Assay kit (V13181, Molecular Probes, ThermoFisher Scientific) was used to evaluate the adherence of monocytes to HUVECs. The procedure used fluorescent dye to label the human monocyte cells, which were then added to culture HUVECs. To assess monocyte adhesion, human monocytic THP-1 cells were labelled with 5 µM of calcein for 30 minutes. The calcein-labelled cells were co-cultured with the

HUVECs, and then incubated for 60 minutes. Relative fluorescence was used as an index of adhesion

RNA Extraction and Synthesis of Single-stranded cDNA

RNA was extracted from the HUVECs using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Single-stranded cDNA was synthesised using a RevertAid Reverse Transcription Kit (ThermoFisher Scientific, USA) following the manufacturer's instructions. A Thermo Scientific Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to quantify and determine RNA concentrations. RNA integrity was also tested by denaturing gel electrophoresis and running an aliquot of the RNA sample on an agarose gel (1% agarose gel was dissolved in 100 ml 1x TAE buffer) stained with ethidium bromide (EtBr). Primer Express Software v3.0 (Applied Biosystems, USA) was used to design the primers for real-time polymerase chain reaction (RT-PCR). All sequences were taken from the National Centre for Biotechnology Information (NCBI). The design of the primers was confirmed using the Basic Local Alignment Search Tool (BLAST). Quantification of mRNA was performed as previously described. The forward and reverse primer pairs are listed as in Table 1. A LightCycler 480 (Roche) was used to perform Real-Time Polymerase Chain Reaction RT-PCR. The method involved a cycling state of 95°C for 5 min, then 45 cycles for 10 s at 95°C, followed by an annealing for 15 seconds at 60 ºC and elongation step for 15 seconds at 72ºC. Using a SYBR Green Master Mix. All values were normalised to the cyclophilin B housekeeping gene [11]. Expression of cyclophilin was not significantly influenced by either LPS treatment or variation in Mg concentration.

Western Blot Analysis

The cells were lysed in 120 µl RIPA (radio-immunoprecipitation assay) buffer (Sigma Aldrich, UK) was added with 5 µl/ml of protease inhibitor cocktail (Calbiochem, USA). The samples were denatured at 90°C for 5 minutes. Then, 30 µg of protein was loaded into each well in 4-10% mini-protean TGX precast polyacrylamide gradient gel. Next, the samples were transferred to a nitrocellulose membrane, and then the membrane was

blocked. The intercellular cell adhesion moluecule-1 (ICAM-1) proteins were detected by using a primary antibody anti-ICAM (Abcam), followed by a secondary antibody to detect the primary antibody. The membranes were then impregnated with enhanced chemiluminescence (ECL) detection (Prime Western Blotting Detection Reagent Kit, GE Healthcare) and visualised by X-ray film exposure.

Human Cytokine Array

The protein concentration of several cytokines in lysed cells was measured using a Human Cytokine Antibody Array Membrane Kit (Abcam), HUVECs were plated onto six-well plates, and the cells were stimulated with 0.5 μg/ml LPS for 4h. At the end of the stimulation time, the cells were lysed in RIPA buffer with protease inhibitor cocktails. The plate was incubated on ice for 30 minutes. Cells were harvested and the array membrane was blocked according to the manufacturer's instructions. The spot signal densities were analysed using Quantity One Analysis Software (Bio-Rad).

Enzyme-linked Immunosorbent Assay (ELISA)

The Human vascular cell adhesion moluecule-1 (VCAM-1) DuoSet kit (R&D Systems, Ltd.) is a quantitative sandwich enzyme immunoassay microplate-based technique. This kit was used to measure VCAM-1 in cell culture supernatants.

Statistical Analyses

Each treatment group comprised four samples with a sample being a single well on a separate culture plate. All data are presented as mean ± standard error of mean [12]. The mRNA expression data were analysed using a two-way analysis of variance (ANOVA; SPSS Statistics 22 software). Data with $P<0.05$ were regarded as statistically significant. Statistical significance between treatment groups was determined using Dunnett's test (1mM magnesium group as reference) as a post-hoc test for the effect of Mg where univariate effects were indicated by the ANOVA. No post-hoc testing was performed where interactions of Mg concentration x LPS treatment were observed. This compared low- and high-Mg concentrations to the control, when variances were homogeneous among the

tested groups. Protein expression data were analysed using a one-way analysis of variance (ANOVA; SPSS Statistics 22 software) with Dunnett's test as a post-hoc test (1mM magnesium group as reference).

Results

The viability of HUVECs in culture was impacted by the concentration of $MgSO_4$ in the medium. Overall, low magnesium conditions resulted in lower cell viability while a significantly greater proportion of viable cells were observed when HUVECs were cultured in high magnesium concentrations, compared to the control 1 mM (Figure 1). After HUVECs were stimulated with 0.1 μg/ml LPS or 0.5 μg/ml LPS for 24 hours, there was a significant loss of viability in both a Mg concentration-dependent and LPS concentration-dependent manner ($P<0.001$). The effect of 48 hours LPS-stimulation on viability varied with Mg concentration (interaction of LPS and Mg concentration, $P=0.018$). Viability of cells grown in 5 mM $MgSO_4$ after treatment with 0.5 μg/ml LPS for 24 and 48 hours was improved relative to the control. Conversely, LPS treatment of HUVECs cultured in 0.1 mM $MgSO_4$ resulted in markedly lower cell viability (Figures 1 a and b). Cell viability was also assessed using the MTT assay method [13] which showed the same trends as identified using trypan blue staining (data not shown).

The effects of varying Mg concentration and inflammatory challenge on the expression of adhesion molecules by HUVECs were evaluated at both the mRNA and protein level. The expression of ICAM-1 and VCAM-1 mRNA was influenced by a significant interaction of Mg concentration and presence of LPS ($P=0.001$, $P<0.001$ respectively). In the absence of LPS, low Mg increased expression of ICAM-1, but not VCAM-1, whilst high Mg had the opposite effect (Figure 2 a and b). Following the treatment of cells with LPS to induce an inflammatory response, both ICAM-1 and VCAM-1 mRNA was significantly increased in the presence of low Mg, compared to the physiological concentration 1mM. In contrast, the higher Mg concentration significantly reduced the mRNA expression for both ICAM-1 and VCAM-1, relative to 1 mM controls (Figure 2 a and b). Western blot analysis showed a significant increase in ICAM-1 protein ($P=0.048$) in response to low magnesium

concentrations (Figure 2 c) in the absence of LPS, but no impact of high magnesium concentration. Concentrations of VCAM-1 in the culture medium significantly increased in magnesium deficient cells that were stimulated with LPS (P=0.032) (Figure 2 d).

Having established that varying Mg concentration could alter the expression of adhesion molecules by HUVECs, we evaluated the possibility that the expression of cytokines in response to inflammatory challenge was also modulated by extracellular magnesium concentration (Table 2). Magnesium had a significant effect on interleukin 8 (IL-8) and monocyte chemotactic protein 1 (MCP-1) mRNA expression in a dose- and LPS-dependent (P=0.004, P<0.001 respectively for magnesium concentration × presence of LPS interaction). In the presence of LPS, cells cultured in low Mg had significantly greater mRNA concentrations for IL-8 and MCP-1 relative to cells cultured in control medium (Table 2). In contrast, a marked decline in IL-8 and MCP-1 mRNA was seen at the high Mg concentration (5 mM) with LPS treatment (Table 2). Mg and LPS also had a significant effect on expression of interleukin 6 (IL-6) mRNA (P=0.002, P<0.001 respectively). A marked decline in IL-6 mRNA (P=0.043) was seen at the highest magnesium concentration 5 mM with LPS treatment compared to control (Table 2). No significant change in the expression of interleukin-1 (IL-1B) and tumour necrosis factor- beta (TNF-β) was found between the magnesium treatment groups and within the same magnesium concentration groups compared to LPS-treated group (Table 2).

Given that measurements of the mRNA expression of selected cytokines showed variation in the inflammatory response with both low and high Mg, additional evidence was sought to establish whether different concentrations of magnesium could modulate the protein concentration of a broad spectrum of inflammatory cytokines. Cytokine proteins were measured using the human cytokine antibody array membrane kit. The array was designed to detect 42 human cytokine proteins; for this study we concentrated on the 18 cytokine proteins actually expressed in HUVECs. The assay confirmed that Mg insufficiency notably enhanced the expression of many cytokines from HUVECs following challenge with LPS, including interleukin-2 (IL-2; P=0.029), interleukin-3 (IL-3; P=0.023), IL-8 (P=0.038),

MCP-1 (P=0.029), growth-related oncogene (GRO; P=0.003) and GROα (P=0.044) compared to the 1mM Mg concentration (Table 3). Conversely, the results showed that the high Mg concentration (5 mM) inhibited the protein concentration of IL-2 (P=0.05) and IL-6 (P=0.01) (Table 3). Consistent with the mRNA expression data magnesium did not affect IL-1 and TNF protein expression. Additionally, no effect of Mg was observed on the cellular concentrations of interleukin-4 (IL4), interleukin-5 (IL5), interleukin-7 (IL7), interleukin-10 (IL10), interleukin-12 (IL12), interleukin-13 (IL13) and interleukin-15 (IL15).

Having demonstrated that low Mg exacerbates the HUVEC response to LPS challenge and that high Mg alleviates this, a further experiment was conducted to demonstrate the functional impact of these effects. Monocyte adhesion was measured through co-incubation of labelled THP-1 cells with HUVECs grown in varying Mg conditions and subject to inflammatory challenge. Adhesion was markedly increased by LPS treatment only in the cells cultured in 5 mM $MgSO_4$ (interaction of LPS and Mg concentration P=0.044). In the presence and absence of LPS, the adhesion of THP-1 cells was significantly higher when HUVECs were cultured in low Mg, compared to the control group. In contrast, treating HUVECs with 5mM Mg significantly inhibited the adhesion of THP-1 cells to HUVECs (Figure 5).

Discussion

The aim of the present study was to investigate whether exposure to Mg- deficient or high Mg concentrations would modulate the response of HUVECs to an inflammatory challenge. The key findings were that low Mg concentrations resulted in an increase in expression of adhesion molecules and inflammatory cytokines, even in the absence of inflammatory stimulus and that this enhanced binding of monocytes to endothelial cells. Providing high concentrations of Mg dampened the response to inflammatory stimulus.

A number of studies have reported decreased proliferation of HUVECs after culturing in low Mg concentrations [9, 10, 14]. The findings of the present study suggest that low Mg is associated with greater cell death, particularly following an inflammatory challenge. In

Mg-deficient cells reduced cell viability was seen at both concentrations of LPS that were used and this can be taken as an indicator of LPS-induced cell death, though further work would be needed to establish whether this was necrosis or apoptosis. In contrast, the high Mg concentration protected the HUVECs from LPS-induced cell death, when stimulated with 0.5 μg/ml LPS for 24 hours. These data are consistent with other studies which have reported that higher Mg concentrations promote HUVEC proliferation [15, 16].

Several studies have reported that stimulating endothelial cells with TNF results in the upregulation of VCAM-1 and ICAM-1 expression [17-20]. Similarly, activating HUVECs with IL-4 results in an increased VCAM-1 expression that is dependent on activated NF-κB binding to the transcription site on the VCAM gene [21]. Increased expression of the adhesion molecules by endothelial cells is one of the steps that initiate atherosclerosis and Cybulsky, *et al.,* [22] found that among mice expressing a low level of VCAM-1, there was a significantly reduced occurrence of early atherosclerosis lesions in the aorta compared to the control group. Humans with high levels of ICAM-1, and VCAM-1 are at greater risk of coronary heart disease [23]. The findings of the present study confirmed that HUVECs grown in 0.1mM magnesium significantly increased ICAM-1 mRNA and protein concentrations and showed sharp and significant increases in ICAM-1 and VCAM-1 expression on exposure to LPS. Rochelsen *et al*., reported that when HUVECs were cultured in 10mM $MgSO_4$ for just 30 minutes before adding LPS, there was suppressive effect on ICAM-1 protein expression (40% less than the control). Adding 10mM $MgSO_4$ concurrently with the LPS lowered the ICAM-1 protein expression to 25% [24]. The current study has shown that culturing HUVECs in high concentrations of Mg (5 mM) similarly decreased gene expression for both ICAM-1 and VCAM-1. We found that the high expression of adhesion molecules in the 0.1 mM Mg condition was associated with greatly increased monocyte adhesion to HUVECs. However, only cells incubated in 5mM Mg showed a significant increase in adhesion with LPS treatment. This is unexplained as it did not relate to either protein of mRNA expression of ICAM-1 and VCAM-1.

Culturing HUVECs in a low Mg concentration and then subjecting the cells to an inflammatory challenge increased the gene and protein expression of IL-8 and MCP-1 relative to the physiological concentration of magnesium. Low Mg concentration also significantly increased the protein expression of GRO, GROα, IL-2 and IL-3. These findings are consistent with the idea that Mg deficiency increases the inflammatory response to LPS and with the report of Ferre *et al*., who showed that low Mg concentrations (0.1 mM) increased the expression of IL-8 protein in HUVECs, but did not affect the expression of TNF-α [25]. To the best of our knowledge there are no other studies examining the effect of low Mg on the protein expression of IL-6, MCP-1, GRO, GROα, IL-2 and IL-3. Whilst Tam *et al*., reported that treatment of pregnant rats with supplemental Mg suppressed the inflammatory response to LPS *in vivo* [26], there are no other studies which have shown the blunting of inflammatory cytokine production by cultured endothelial cells. Our study therefore provides a new insight into the potential role for magnesium in limiting the inflammatory cascade.

Low Mg status has been shown to be related to inflammation *in vivo* [15, 27, 28]. The Harvard Nurses Study found a negative relationship between Mg intake and the level of inflammatory response in plasma [29]. Similarly, the Nurse's Health Study showed that women who had a high Mg intake had lower plasma inflammatory markers, such as C-reactive protein (CRP) and E-selectin [30]. Furthermore, Sugimoto, *et al.,* [31] showed that the inflammatory response to LPS was decreased in human umbilical cord blood following treatment with $MgSO_4$. Similarly, rat placentas collected 4 hours after injection of LPS with $MgSO_4$ showed reduced expression of IL-6, TNF-1 and MCP-1 [32]. Malpuech-Brugère*, et al.,* [33] found that rats fed a Mg-deficient diet for four days had higher IL-6 in their plasma compared to controls. The present study is therefore consistent with a broad body of literature including an in *vitro* study in which microvascular endothelial cells cultured in low magnesium (0.1 mM) increased the production of VCAM-1, IL-1 and IL-6 in response to LPS challenge [15]. The mechanisms for the anti- inflammatory effect of

Mg are not clear [31, 33-35], but we have recently demonstrated involvement of the toll-like receptor 4 in mediating the magnesium-LPS interaction [36].

In summary, these data show that low Mg concentrations enhance the inflammatory response to LPS, eliciting a specific profile of cytokine expression. Supplementing cells with Mg appears to suppress the inflammatory response and protect the endothelium from both damage. The findings add weight to the argument that Mg is an important determinant of vascular endothelial cell function. The capacity for Mg to modulate inflammatory responses in the vasculature may, at least partly, explain why Mg deficiency is associated with increased risk of atherosclerotic cardiovascular disease.

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Figure legends

Figure 1: **Dependence of HUVECs viability on $MgSO_4$ concentration as determined by the trypan blue exclusion assay.** Data are shown as mean ± SEM for n=4. A: Viable cells stimulation for 24 hours. Two-way ANOVA showed a significant interaction of magnesium concentration × LPS concentration (P <0.001). There was significant effect of magnesium concentration (P <0.001) and LPS (P <0.001). B: Viable cells Stimulation for 48 hours. Two-way ANOVA showed a significant interaction of magnesium concentration × LPS concentration (P <0.018). There was significant effect of magnesium concentration (P <0.002) and LPS (P <0.001).

Figure 2: **Effect of different concentrations of magnesium on adhesion molecule expression.** Data are shown as mean±SEM for n=4. **A: Expression of ICAM-1 mRNA.** Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P = 0.001). There was a significant effect of magnesium concentration (P< 0.001), and presence of LPS (P<

0.001). **B: Expression of VCAM-1 mRNA.** Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P< 0.001). There was a significant effect of magnesium concentration (P< 0.001), and presence of LPS (P< 0.001). C: **Expression of ICAM-1 protein.** One way ANOVA showed significant effect of magnesium concentration (P=0.038), with a marked increase in ICAM-1 protein concentration in low magnesium concentration (P=0.048, Dunnett t). Data are shown as mean±SEM for n=3. * indicates P<0.05, compared to 1mM $MgSO_4$ in the same treatment (Dunnett t). **D, Expression of VCAM-1 protein.** Data are shown as mean±SEM for n=4. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS. There was a significant effect of magnesium concentration (P= 0.001), and presence of LPS (P = 0.021).

Figure 3: THP-1 adherence to HUVECs. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P = 0.044). There was a significant effect of magnesium concentration (P< 0.001), and presence of LPS (P = 0.047).

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427 Table 1 Primer sequences used for RT-PCR

Symbol	Gene name	Forward primer	Reverse primer
IL-8	Interleukin 8	ACCGGAAGGAACCATCTCACT	ATCAGGAAGGCTGCCAAGAG
TNF-Beta	Tumor necrosis factor Beta Lymphotoxin Alpha	TGTTGGCCTCACACCTTCAG	TGCTGTGGGCAAGATGCAT
IL-1B	Interleukin 1, beta	TCTGCCCAGTTCCCCAACT	TGGTCCCTCCCAGGAAGAC
ICAM-1	Intercellular adhesion molecule	TCCCCCCGGTATGAGATTG	GCCTGCAGTGCCCATTATG
VCAM	Vascular cell adhesion molecule	GCAAGGTTCCTAGCGTGTAC	GGCTCAAGCATGTCATATTCAC
IL-6	Interleukin 6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT
MCP-1	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2)	CGCCTCCAGCATGAAAGTCT	GGAATGAAGGTGGCTGCTATG
PPI	Cyclophilin B	GGAGATGGCACACAGGAGGAAA	CGTAGTGCTTCAGTTTGAAGTTCTCA

428

429

Table 2 Effect of different concentrations of magnesium on cytokine mRNA expression

Genes		0.1 mM $MgSO_4$	1mM $MgSO_4$	5mM $MgSO_4$	*P* value Mg	*P* value LPS	*P* value Mg× LPS interaction
IL-8	- LPS	0.48 ± 0.068	0.33 ± 0.04	0.31 ± 0.11	0.001	0.004	< 0.001
	+ LPS	2.6 ± 0.44	1.4 ± 0.27	0.63 ± 0.06			
IL-6	- LPS	1.2 ± 0.08	1.15 ± 0.2	0.97 ± 0.14	0.002	0.053	< 0.001
	+ LPS	2.6 ± 0.37	2.2 ± 0.07	1.5 ± 0.09			
MCP-1	- LPS	0.32 ± 0.015	0.21 ± 0.029	0.18 ± 0.001	< 0.001	< 0.001	< 0.001
	+ LPS	2.5 ± 0.32	1.4 ± 0.23	0.053 ± 0.04			
IL-1β	- LPS	0.15 ± 0.027	0.11 ± 0.026	0.12 ± 0.028	0.31	0.46	0.19
	+ LPS	0.17 ± 0.04	0.12 ± 0.03	0.21 ± 0.05			
TNF-β	- LPS	0.12 ± 0.02	0.097 ± 0.04	0.21 ± 0.01	0.66	0.87	0.94
	+ LPS	0.18 ± 0.07	0.11 ± 0.02	0.13 ± 0.06			

Data are shown as mean ± SEM for n=4 observations.

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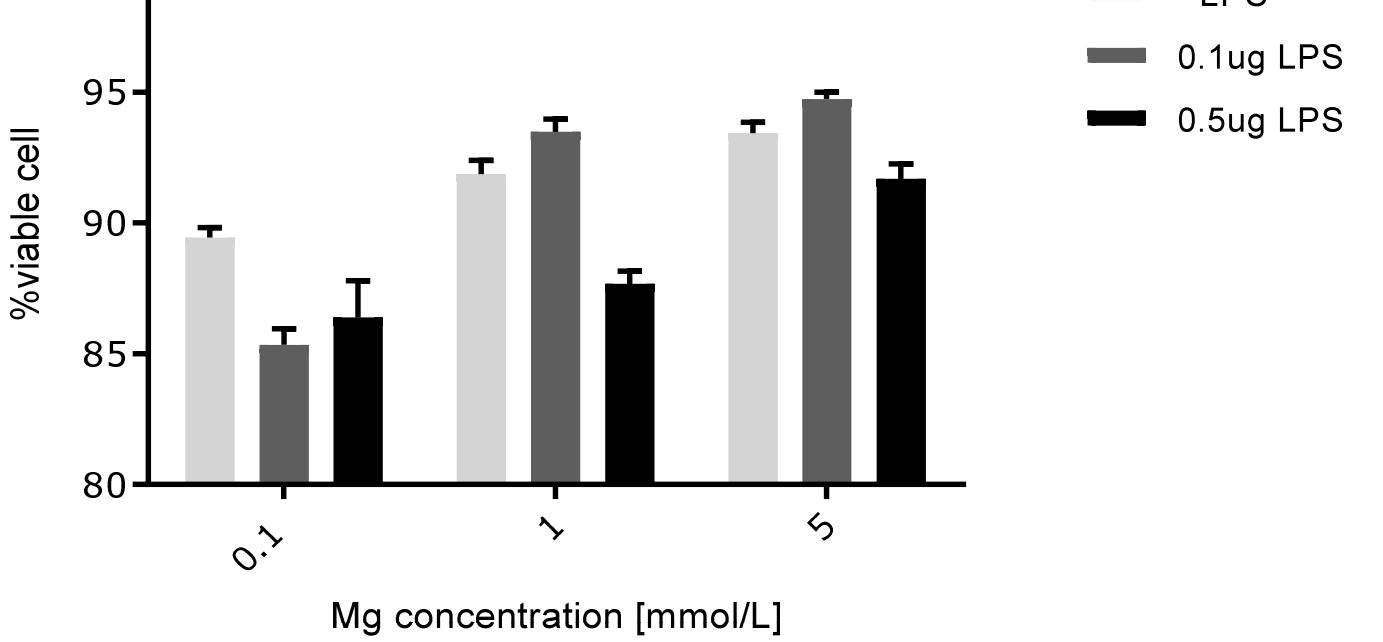
457

458 Table 3 Effect of different concentrations of magnesium on cytokine protein concentration

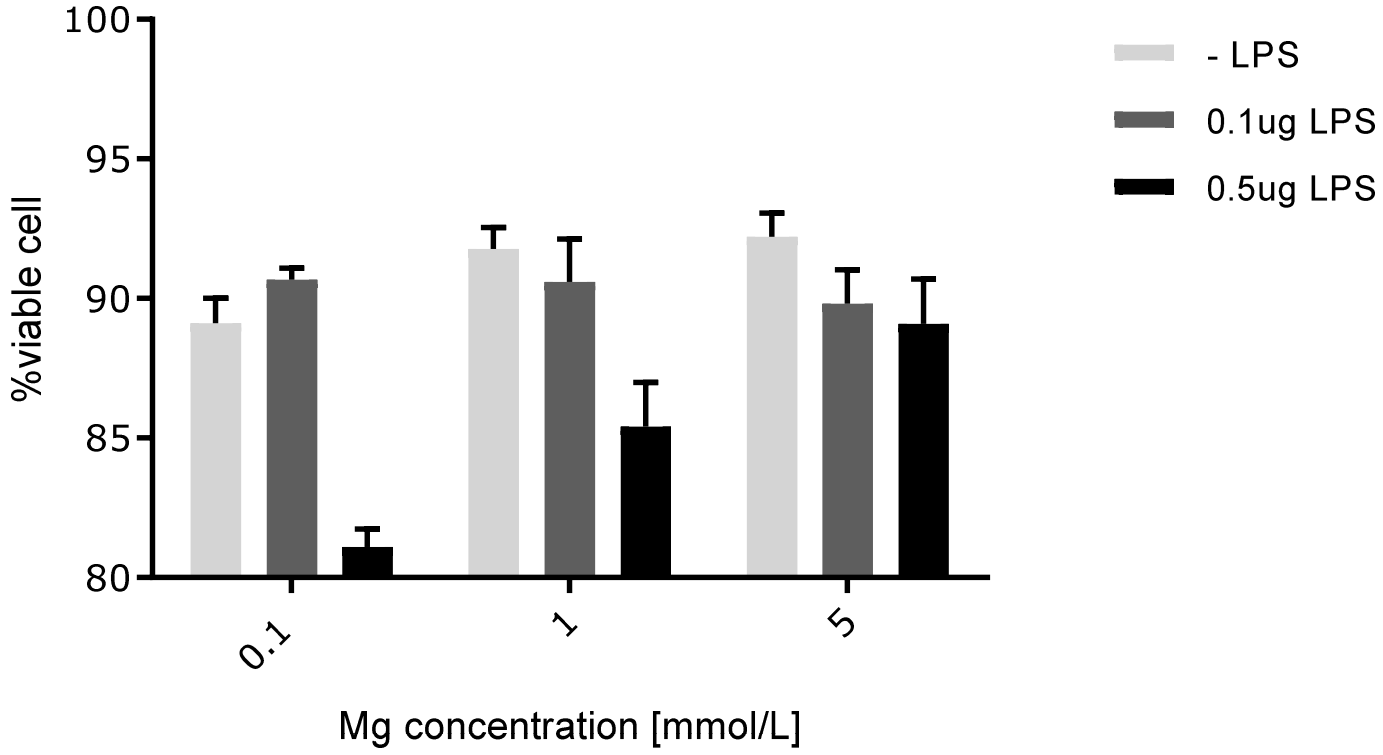
Genes	0.1 mM $MgSO_4$	1mM $MgSO_4$	5mM $MgSO_4$	*P* value 0.1mM vs 1mM	*P* value 5mM vs 1mM
IL-1 α	0.067 ± 0.034	0.03 ± 0.009	0.052 ± 0.022	0.24	0.55
IL-1 β	0.17 ± 0.02	0.12 ± 0.055	0.11 ± 0.05	0.65	0.99
IL-2	0.08 ± 0.003	0.049 ± 0.007	0.022 ± 0.007	0.029	0.05
IL-3	0.27 ± 0.02	0.16 ± 0.03	0.13 ± 0.019	0.023	0.5
IL-4	0.063 ± 0.025	0.042 ± 0.012	0.037 ± 0.013	0.5	0.65
IL-5	0.097 ± 0.016	0.07 ± 0.01	0.029 ± 0.007	0.26	0.096
IL-6	0.042 ± 0.001	0.036 ± 0.006	0.011 ± 0.002	0.49	0.01
IL-7	0.138 ± 0.04	0.066 ± 0.016	0.033 ± 0.01	0.38	0.21
IL-8	0.6 ± 0.12	0.28 ± 0.05	0.15 ± 0.003	0.038	0.42
IL-10	0.12 ± 0.02	0.094 ± 0.02	0.089 ± 0.05	0.81	0.99
IL-12	0.3 ± 0.019	0.23 ± 0.07	0.15 ± 0.04	0.56	0.42
IL-13	0.074 ± 0.018	0.044 ± 0.013	0.038 ± 0.018	0.95	0.24
IL-15	0.29 ± 0.05	0.14 ± 0.007	0.2 ± 0.03	0.046	0.44
MCP-1	0.71 ± 0.036	0.47 ± 0.03	0.31 ± 0.07	0.029	0.12
GRO	0.63 ± 0.03	0.23 ± 0.036	0.18 ± 0.075	0.003	0.79
GRO-α	0.3 ± 0.007	0.14 ± 0.049	0.11 ± 0.045	0.044	0.78
TNF-α	0.061 ± 0.035	0.052 ± 0.017	0.07 ± 0.027	0.92	0.59
TNF-β	0.13 ± 0.019	0.13 ± 0.03	0.11 ± 0.037	0.99	0.94

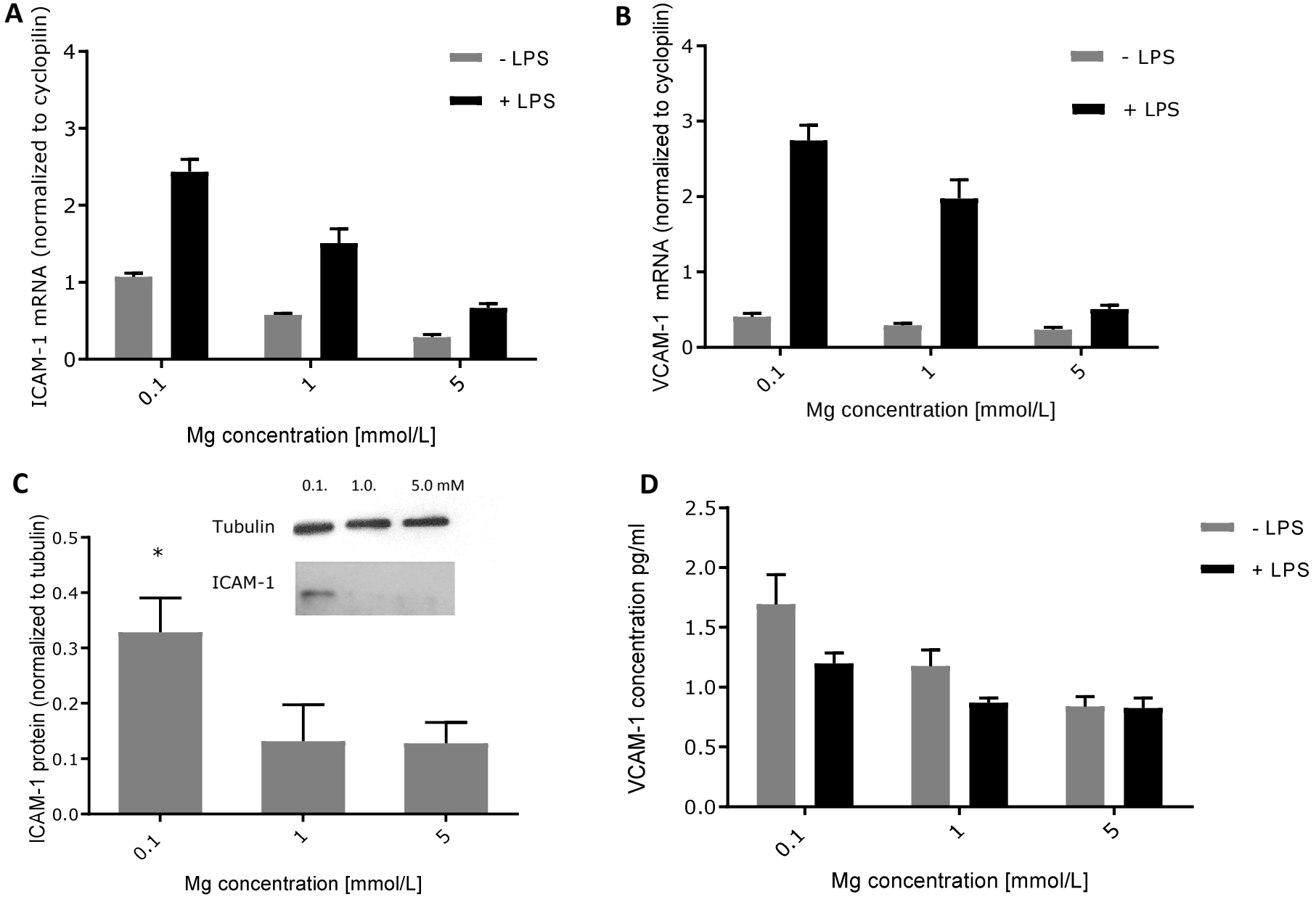
459 Data are shown as mean ± SEM for n=3 observations.

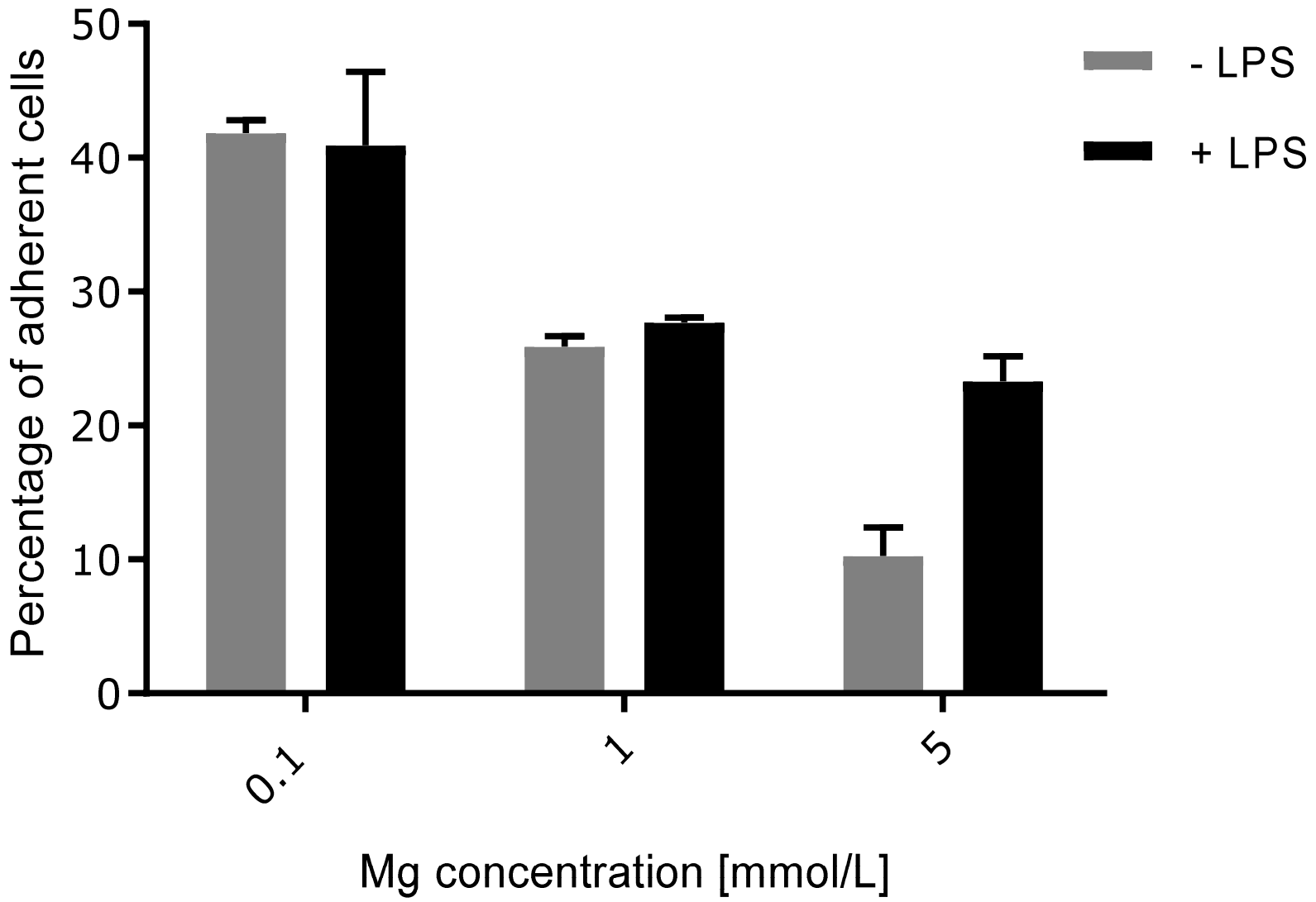
460


95
90
85
80
%viable cell
0.1
1
5
Mg concentration [mmol/L]
0.1ug LPS
0.5ug LPS

B


100
95
90
85
80
%viable cell
0.1
1
5
Mg concentration [mmol/L]
- LPS
0.1ug LPS
0.5ug LPS


A
ICAM-1 mRNA (normalized to cyclopilin)
- LPS
+ LPS
0
1
2
3
4
0.1
1
5
Mg concentration [mmol/L]
B
VCAM-1 mRNA (normalized to cyclopilin)
- LPS
+ LPS
0
1
2
3
4
0.1
1
5
Mg concentration [mmol/L]
C
0.1.
1.0.
5.0 mM
Tubulin
ICAM-1
ICAM-1 protein (normalized to tubulin)
*
0.0
0.1
0.2
0.3
0.4
0.5
0.1
1
5
Mg concentration [mmol/L]
D
VCAM-1 concentration pg/ml
- LPS
+ LPS
0.0
0.5
1.0
1.5
2.0
2.5
0.1
1
5
Mg concentration [mmol/L]


Percentage of adherent cells
50
40
30
20
10
0
- LPS
+ LPS
0.1
1
5
Mg concentration [mmol/L]